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ORIGINAL ARTICLE

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The influence of intact-plant and excised-leaf bioassay designs on volicitin- and jasmonic acid-induced sesquiterpene volatile release in *Zea mays*

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Abstract Induced plant responses to insect attack include the release of volatile chemicals. These volatiles are used as host-location signals by foraging parasitoids, which are natural enemies of insect herbivores. A plant's response to herbivory can be influenced by factors present in insect oral secretions. Volicitin (*N*-(17-hydroxylinolenoyl)-L-glutamine), identified in beet armyworm (*Spodoptera exigua*) oral secretions, stimulates volatile release in corn (*Zea mays* L.) seedlings in a manner similar to beet armyworm herbivory. Volicitin is hypothesized to trigger release of induced volatiles, at least in part, by modulating levels of the wound hormone, jasmonic acid (JA). We compare the sesquiterpene volatile release of damaged leaves treated with aqueous buffer only or with the same buffer containing volicitin or JA. Leaves were damaged by scratching with a razor and test solutions were applied to the scratched area. The leaves were either excised from the plant or left intact shortly after this treatment. Plants were treated at three different times (designated as Evening, Midnight, and Morning) and volatiles were collected in the subsequent photoperiod. JA and volicitin treatments stimulated the release of volatile sesquiterpenes, namely β-caryophyllene, (*E*)-α-bergamotene, and (*E*)-β-farnesene. In all cases, JA stimulated significant sesquiterpene release above mechanical damage alone. Volicitin induced an increase in sesquiterpene volatiles for all excised-leaf bioassays and the Midnight intact plants. Volicitin treatments in the Evening and Morning intact plants produced more sesquiterpenes than the untreated controls, while mechanical damage alone produced an intermediate response that did not differ from either treatment group. Excised leaves produced a 2.5- to 8.0-fold greater volatile response than similarly treated intact plants. Excision also altered the ratio of JA-and volicitin-induced sesquiterpene release by preferentially increasing (*E*)-β-farnesene levels relative to β-caryophyllene. The inducibility of volatile release varied with time of treatment. On average, sesquiterpene release was highest in the Midnight excised leaves and lowest in the Morning intact plants. The duration of induced volatile release also differed between treatments. On average, JA produced a sustained release of sesquiterpenes over time, with over 20% of the combined sesquiterpenes released in the third and final volatile collection period. In contrast, less than 8% of the combined sesquiterpenes induced by volicitin were emitted during this period. The large quantitative differences between intact plants and detached leaves suggest that the results of assays using excised tissues should be cautiously interpreted when considering intact-plant models.

Keywords Bioassay (excised vs. intact tissues) · Jasmonic acid · *Spodoptera* (volicitin) · Plant volatiles · Volicitin · *Zea* (herbivory)

Abbreviations BAW: beet armyworm · C/F: β-caryophyllene/(*E*)-β-farnesene · JA: jasmonic acid · OS: oral secretion · volicitin: (*N*-(17-hydroxylinolenoyl)-L-glutamine)

Introduction

In response to insect herbivory, many plants have been shown to rely upon dynamic defensive chemistry and the attraction of the herbivore's natural enemies. Wound-induced increases in toxic or deterrent metabolites have been widely described in plants (Karban and Baldwin 1997). In most cases, increased pools of alkaloids and terpenoids remain stably sequestered for many weeks (Gershenzon et al. 1993; Baldwin et al. 1994). Plant responses to insect herbivory also include the rapid

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biosynthesis and release of volatiles (Paré and Tumlinson 1997). An alternative to the accumulation of direct toxins, these plant-produced terpenoid- and lipoxygenase-derived volatiles can act as reliable signals indicating the presence of potential hosts and prey for respective parasitoids and predators (Vet and Dicke 1992; Turlings et al. 1995).

Many herbivore-induced plant responses are triggered by tissue damage. For example, increased nicotine production in wild tobacco displays a direct quantitative relationship to the amount of leaf damage and resulting increases in the wound hormone, jasmonic acid (JA) (Baldwin et al. 1997). However, plant perception and response to herbivory can demonstrate specificity beyond a generalized wound response. In corn (*Zea mays*) seedlings, only trace levels of volatile terpenoids are released following mechanical damage. Following leaf damage by armyworm (*Spodoptera* sp.) feeding, corn exhibits greatly enhanced volatile release, including the sesquiterpenes β-caryophyllene, (*E*)-α-bergamotene, and (*E*)-β-farnesene (Turlings et al. 1990). Beet armyworm (BAW; *Spodoptera exigua*) oral secretions (OS), applied to mechanical-damage sites, are sufficient to mimic the plant response to caterpillar feeding (Turlings et al. 1990). Volicitin (*N*-(17-hydroxylinolenoyl)-L-glutamine) has been isolated and identified in BAW OS and is a potent elicitor of volatile biosynthesis (Alborn et al. 1997). Additional N-acylamino acids are now known to occur in the OS of numerous insect species (Pohnert et al. 1999; Alborn et al. 2000). Quantitative and qualitative differences in elicitors from insect OS may partly explain why plant volatile responses and related parasitoid attraction differ following herbivore damage of closely related insects (De Moraes et al. 1998). Understanding the biochemical mechanisms and ecological specificities of these interactions will be crucial to enhancing the benefit of these interactions in agricultural settings.

Elicitors from insect OS, such as volicitin, have been hypothesized to interact with the octadecanoic acid pathway and influence volatile production by modulating JA levels and ultimately gene expression in plants (Hopke et al. 1994; Paré and Tumlinson 1999). Thus far, a detailed analysis of plant volatile responses to both JA and volicitin has not been reported. The growing interest in plant volatiles has been accompanied by a diverse array of bioassay designs, inducing treatments, volatile collection periods, and analytical methodologies. Excised plant and leaf assays, which are routinely used to examine induced volatile release, have provided easy and reproducible means to monitor the activity of bioactive fractions and thus have enabled the isolation and identification of elicitors (Alborn et al. 1997). However, elicitors from leaf-feeding caterpillars would not normally enter the plant through the xylem stream of an excised stem. Few, if any, studies have critically examined the potential differences between the volatile responses of intact and excised plants. This makes it difficult to compare results from experiments that utilize intact plants (Röse et al. 1996; Turlings et al. 1998; Halitschke et al. 2000) and those which measure volatiles from tissues several days after excision (Takabayashi et al. 1991; Dicke and Dijkman 1992; Koch et al. 1999).

From laboratory and field perspectives, we believe there is value in understanding bioassays using both excised tissues and more agriculturally relevant intact plants when considering plant signal transduction from perception to response. To address this current gap we have examined mechanical damage, JA and volicitin treatment effects on sesquiterpene release in corn seedlings, and have quantified differences between intact-plant and excised-leaf bioassays while varying the treatment time prior to volatile collection.

Materials and methods

Plant growth conditions

Seeds of *Zea mays* L. cv. Delprim were acquired from Delley Seeds and Plants (Delley, Switzerland) and germinated in potting soil (MG500SC; Scotts-Sierra Horticultural Products Co, Marysville, Ohio, USA). After 6 days of growth, seedlings were removed from the soil, the roots rinsed with water, and transferred to 1-l hydroponic containers. The nutrient solution consisted of 1 mM KNO_3, 0.75 mM $MgSO_4 \cdot 7H_2O$, 0.5 mM $Ca(NO_3)_2 \cdot 4H_2O$, 0.5 mM NH_4NO_3, 0.5 mM KH_2PO_4, 0.25 mM NaCl, 0.25 mM K_2SO_4, 60 μM Fe-Na EDTA, 50 μM H_3BO_3, 15 μM $MnCl_2 \cdot 4H_2O$, 2 μM $ZnSO_4 \cdot 7H_2O$, 0.25 μM $CuSO_4 \cdot 5H_2O$, and 0.2 μM $Na_2MoO_4 \cdot 2H_2O$. All plants were maintained in a 12-h photoperiod with 350 μmol quanta m^{-2} s^{-1} of photosynthetically active radiation, 70% relative humidity and a temperature cycle of 22 °C/26 °C (night/day). Volatile analysis was performed on 10-day-old plants that contained three expanded leaves.

Leaf treatments and bioassay designs

The four leaf treatments were: (i) untreated controls, leaf damage plus (ii) aqueous buffer (50 mM NaH_2PO_4; pH 8.0), and leaf damage plus the same buffer containing either (iii) volicitin (5 nmol/plant) or (iv) JA (30 nmol/plant). For the damage protocol, each of the three leaves received two superficial damage sites using a razor to scratch the abaxial surface of the leaves perpendicular to but not including the midrib vasculature. The damage sites (2 mm×10 mm) were approximately equidistant between the base and tip of the leaf but laterally staggered by 2 cm with one on each side of the midrib. This treatment disrupted the waxy cuticle and epidermal cells and allowed applied buffer solutions to cling to the leaf surface. A total of 18 μl of buffer was distributed evenly between the six damage sites on each plant immediately after wounding. The quantities of volicitin and JA used were previously determined to reproducibly stimulate volatile release over a wide range of conditions (data not shown). JA- and volicitin-induced volatile release could not be readily compared at equal treatment levels as 5 nmol/plant of JA does not stimulate sesquiterpene volatiles from intact plants and 30 nmol/plant of volicitin is entirely unrealistic given the amounts present in BAW OS (Alborn et al. 1997; and data not shown). Twenty minutes after the damage treatments, leaves from designated excised groups were cut at the base of the petiole and placed into 4-ml vials containing purified H_2O. Intact plants were not manipulated further. All leaf treatments were performed either 20 min prior to the beginning, middle, or end of the dark cycle; these treatment times are designated as Evening, Midnight, and Morning, respectively.

Volatile collection

To minimize desiccation, root systems of intact plants were wrapped in wet cotton while the bases of excised leaves remained in vials with H_2O. Intact plants and excised leaves were placed in tapered glass chambers (4.5 cm i.d., 37 cm long, 550 ml volume; Analytical Research Systems, Gainesville, Fla., USA) and assayed under their original lighting conditions. Collection of volatiles follows from Turlings et al. (1991); clean humidified air was passed through the chambers (550 ml/min) and volatiles were trapped on 50 mg Super Q (80/100 mesh; Alltech, Deerfield, Ill., USA) during three 4-h periods (0–4, 4–8, 8–12 h) in the subsequent 12-h light cycle. Super Q traps were eluted with 150 µl dichloromethane, and 400 ng of nonyl acetate (in 5 µl of dichloromethane) was added as an internal standard. This experiment ($n = 6$) compares four leaf treatments, two bioassay designs (intact plant and excised leaf), three treatment times, and three volatile collection periods. Volatile collection was limited to 24 plants per day; thus a total of 6 separate trials were combined to form the final data set (432 samples).

Identification and quantification of volatiles

Gas chromatography-mass spectrometry (GC-MS) was completed on a Hewlett-Packard (HP) 6890 gas chromatograph (He carrier gas; 0.7 ml min^{-1}; splitless injector 240 °C, injection volume 2 µl) with an HP-5MS column (5% phenyl methyl siloxane; 30 m long, 250 µm i.d., 0.25 µm film thickness) with the temperature programmed from 40 °C (1 min hold) at 10 °C min^{-1} to 240 °C (hold for 15 min). The CG was coupled to an HP 5973 quadrupole-type mass selective detector with transfer line, source, and quadrupole temperatures of 230 °C, 230 °C and 150 °C, respectively. Ions were generated at a 70 eV potential and scanned at a range of 30–500 amu. The dominant sesquiterpenes including β-caryophyllene, (*E*)-α-bergamotene, and (*E*)-β-farnesene were identified by comparison of retention times with authentic standards and by comparison of mass spectra with Wiley and NIST libraries. Quantification of volatiles was performed on an HP 6890 gas chromatograph (carrier gas; He at 1.2 ml min^{-1}; splitless injector, temperature 220 °C, injection volume 1 µl) with a Quadrex (New Haven, Conn., USA) 007-series fused silica capillary (methyl silicone; 15 m long, 250 µm i.d., 0.25 µm film thickness) with temperatures programmed from 40 °C (0.5 min hold) at 12 °C min^{-1} to 180 °C then 220 °C (2.0 min hold for the post run). Signals were captured with a flame ionization detector (250 °C).

Statistical analysis

Analyses of variance (ANOVAs) were performed on the combined sesquiterpenes, β-caryophyllene/(*E*)-β-farnesene ratios and sesquiterpene release (percent of total). Significant treatment effects were investigated when the main effects of the ANOVA were significant ($P < 0.05$). Where appropriate, Tukey tests were used to correct for multiple comparisons between intact and excised bioassays and within treatment times and volatile correction periods. Student *t*-tests were used to detect changes in excision-induced sesquiterpene ratios for each type and time of treatment. The analysis was accomplished with JMP 3.0 statistical discovery software (SAS Institute Inc., Cary, N.C., USA).

Results

Bioassay design influences the quantity of induced sesquiterpenes

In both excised-leaf and intact-plant bioassays, volicitin and JA stimulated increases in sesquiterpene volatiles compared to mechanical damage alone (Fig. 1A–F). However, compared to the intact-plant bioassay, excision greatly increased both volicitin- and JA-induced volatile release (Fig. 2A, B). In all cases, JA increased volatile release above mechanically damaged plants (Fig. 2A, B). Volicitin induced significantly more sesquiterpenes than damage alone in all excised-leaf bioassays and intact plants treated at Midnight. Volatile responses from intact plants mechanically damaged in either the Evening or Morning were intermediate and not significantly different from either the control or the volicitin treatment (Fig. 2B). In all cases, volicitin-treated plants produced greater quantities of sesquiterpenes than the undamaged controls (Fig. 2A, B). With one exception, mechanical damage alone did not significantly induce volatile release above the undamaged controls (Fig. 2A, B). Excision did not alter sesquiterpene release in undamaged control leaves (Fig. 2A, B); however, an important interaction was detected between excision, damage, and elicitation. In the Midnight excised treatment, damage alone induced a 4.1-fold greater volatile response than similarly treated intact plants (Fig. 2A, B). In the Evening, Midnight and Morning, excision increased the volicitin-induced sesquiterpene release by 2.5-, 8.0-, and 4.9-fold respectively and likewise the JA-induced sesquiterpene release by 2.5-, 6.0-, and 3.6-fold respectively, compared to similarly treated intact plants. Thus excision can increase a plant's response to damage alone but a much greater volatile response occurs with the interaction of leaf damage, elicitation, and excision.

Time of treatment affects release of induced sesquiterpenes

Both intact plants (Fig. 1A–C) and excised leaves (Fig. 1D, E) demonstrated a maximum JA-induced sesquiterpene release during periods 1 and 2 when treated in the Evening and at Midnight. However, the peak release shifted to periods 2 and 3 when the plants were treated in the Morning. For a majority of the treatments, (*E*)-β-farnesene was the major induced sesquiterpene released in time period 1. The exception was intact plants treated in the Evening, where β-caryophyllene was the dominant sesquiterpene. In contrast, caryophyllene was the dominant sesquiterpene induced by JA and volicitin in period 3 (Fig. 1A–F). Volicitin-induced sesquiterpene volatiles peaked in period 1, except for the Morning excised group where release was sustained in period 2. Time of elicitation also affected the combined sesquiterpene release in the following photoperiod. Volicitin and JA stimulated the largest volatile response in excised leaves that were treated at Midnight (Fig. 2A). This result was largely due to (*E*)-β-farnesene levels in periods 1 and 2 (Fig. 1E). In contrast, intact plants treated in the Morning with volicitin did not significantly increase sesquiterpene emission above damage alone (Fig. 2B).

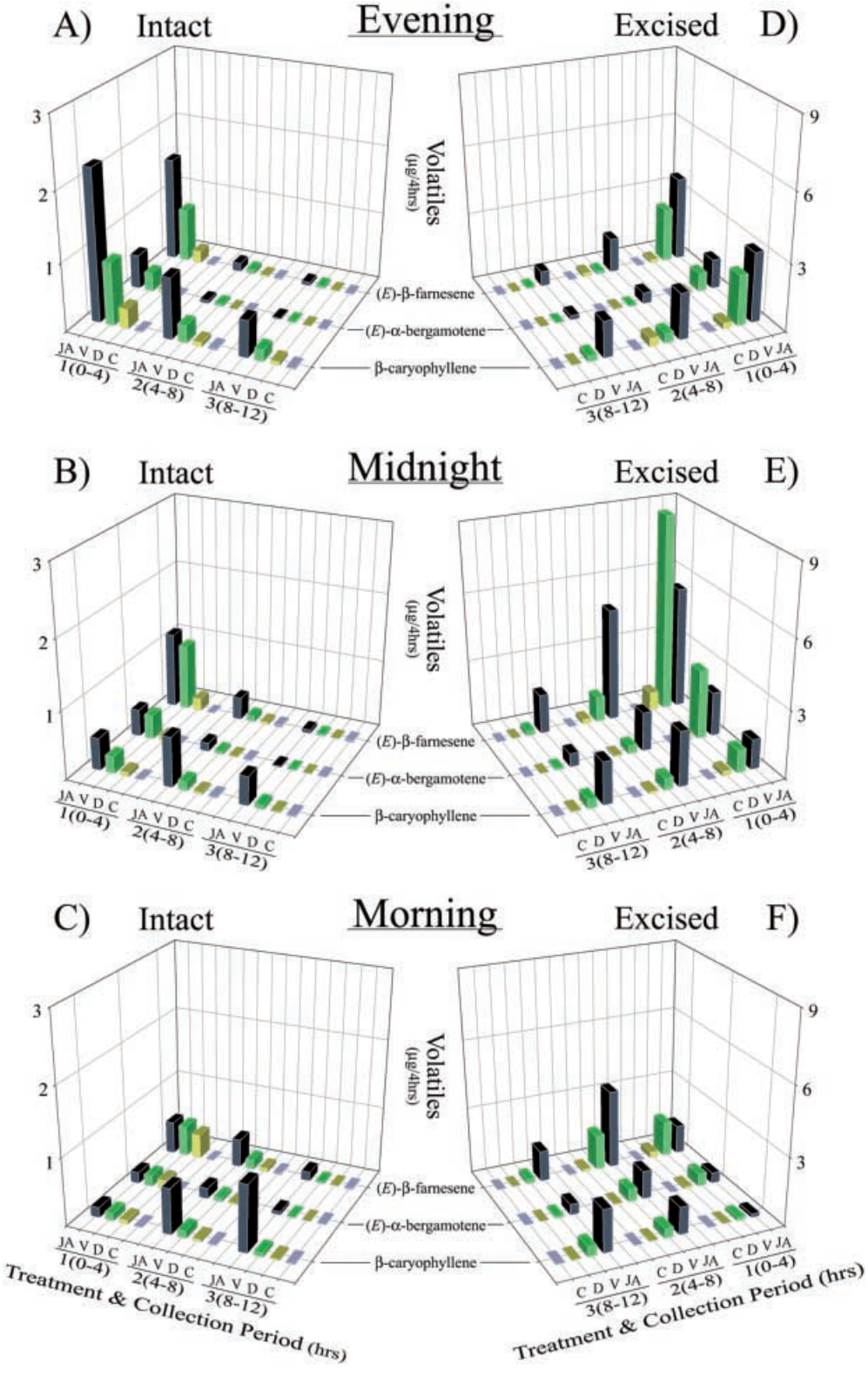

Fig. 1A–F Average ($n = 6$) induced sesquiterpene production [(*E*)-*β*-farnesene, (*E*)-*α*-bergamotene, *β*-caryophyllene] from intact and excised corn (*Zea mays* cv. Delprim) seedlings following no treatment (*C*), damage plus aqueous buffer only (*D*), or damage plus aqueous buffer containing volicitin (*V*) or jasmonic acid (*JA*). Treatments were conducted in the Evening, Midnight, or Morning and volatiles collected in three 4-h periods (*1* 0–4 h, *2* 4–8 h, *3* 8–12 h) during the following photoperiod. Within the three treatment times, results for intact-plant and excised-leaf bioassay are arranged as mirror images for comparative purposes (Note: change in scale **A–C** vs. **D–F**)

Excision changes sesquiterpene ratios

Both excision and time of treatment significantly altered the ratio of sesquiterpenes released following JA and volicitin treatments. Over the 12-h photoperiod, excision reduced the relative amount of *β*-caryophyllene to (*E*)-*β*-farnesene (C/F) released by JA-treated leaves between 2.0- and 2.9-fold (Fig. 3A). Similarly, excision reduced the C/F ratio in volicitin-treated leaves between 1.3-and 2.1-fold. Interestingly, excision did not alter the

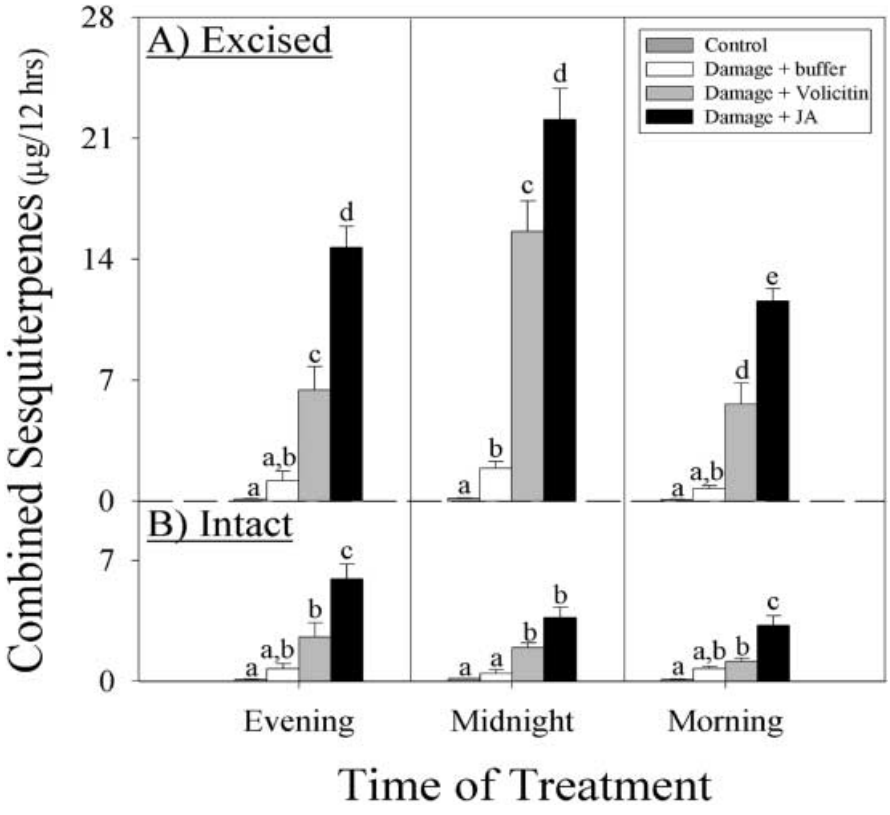

Fig. 2 Mean (+SE; $n=6$) combined sesquiterpenes (µg/12 h) from excised leaves (**A**) and intact (**B**) corn seedlings treated in the Evening, Midnight or Morning. Different letters (*a*, *b*, *c*, *d*, *e*) represent significant differences within each time of treatment ($P>0.05$, Tukey correction for multiple comparisons)

C/F ratio in the mechanical-damage group (Fig. 3C). Within each time of treatment, the reduced C/F ratio caused by excision is driven by an increase in (*E*)-*β*-farnesene relative to *β*-caryophyllene (Fig. 1A–F). Across treatment times, there is a general trend for decreased C/F ratios from Evening to Midnight and Morning treatments.

Kinetics of volatile release

Independent of bioassay design and time of treatment, JA stimulated an increase in volatile sesquiterpenes over the entire 12 h volatile collection period (Fig. 1A–F). In contrast, while volicitin induced the largest single volatile increase, release over time did not mirror the sustained volatile emission evident from JA-treated plants (Fig. 1A–F). For example, in the Midnight excised group, volicitin stimulated (*E*)-*β*-farnesene release of 8.8 and 1.1 µg/4 h in the first and second periods, respectively, while JA stimulated (*E*)-*β*-farnesene release of 5.4 and 5.0 µg/4 h during these same two periods (Fig. 1E). On average, between bioassay design and treatment times, damaged plants treated with either buffer or volicitin released 68% and 72% of the 12-h total of sesquiterpenes in the first volatile collection period, respectively (Fig. 4). Likewise, sesquiterpene volatiles released by these same plants in the third period accounted for less than 10% of the total emitted. In contrast, JA-induced volatile release was more evenly distributed, with 44.6%, 34.3%, and 21.1% of the total sesquiterpenes in the first, second and third collection periods, respectively (Fig. 4). As expected, low levels of sesquiterpene volatile emission from control plants were nearly uniform between collection periods and ranged between 29.8% and 37.5% (Fig. 4).

Discussion

Insect herbivory is known to induce production and release of volatile terpenes in a wide range of plant taxa, presumably through the activation of plant defense signaling pathways (Paré and Tumlinson 1999). Many studies of induced plant volatiles have introduced elicitors into plants by placing cut stems into aqueous test solutions (Dicke et al. 1993; Turlings et al. 1993; Boland et al. 1995) or have used insect herbivory and oral secretions (OS) on intact plants (Röse et al. 1996; Halitschke et al. 2000). Despite the fact that much of the published work is based on research with either intact or excised plants, bioassay design has been given very little attention and few detailed comparisons have been made between bioassay types and inducing treatments. In this study, we demonstrate that intact-plant and excised-leaf bioassays can greatly differ in both the total amount and ratios of induced sesquiterpenes released. Excision increased the JA- and volicitin-induced sesquiterpene volatiles between 2.5- to 8.0-fold above similarly treated intact plants. Excision also altered the ratios of sesquiterpenes produced by promoting induced (*E*)-*β*-farnesene release and thus lowering the relative

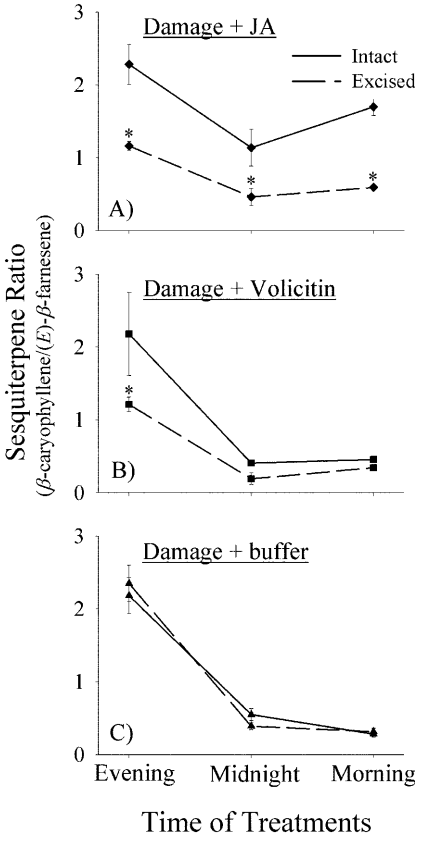

Fig. 3 Mean (±SE; $n=6$) sesquiterpene ratios (β-caryophyllene/(E)-β-farnesene) of excised leaves (*dotted line*) and intact (*solid line*) corn seedlings damaged and treated with JA (**A**), volicitin (**B**), or buffer (**C**) during the Evening, Midnight or Morning. *Asterisks* (*) denote significant differences within each graph and time of treatment ($P<0.05$, students t-test)

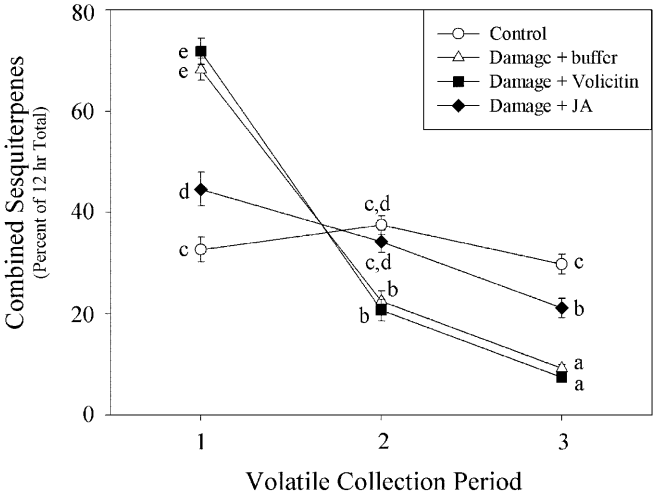

Fig. 4 Mean (±SE; $n=32$) percent of total (12 h) sesquiterpenes released during volatile collection periods *1* (0–4 h), *2* (4–8 h) and *3* (8–12 h) from control corn seedlings (*open circles*) or those damaged and treated with buffer (*open triangles*), volicitin (*filled squares*), or JA (*filled diamonds*). Bioassays (intact plants/excised leaves) and treatment times (Evening, Midnight, Morning) were combined to generate this average. Symbols not sharing the same letters (*a*, *b*, *c*, *d*, *e*) represent significant differences ($P<0.05$, Tukey correction for multiple comparisons)

amount of β-caryophyllene present. The intact plants and excised leaves also differed in response to JA and volicitin depending on the time of treatment. Excised leaves produced the greatest volatile response when treated in the middle of the dark cycle (Midnight), while intact plants displayed little or no response to volicitin when treated at the beginning of the light cycle (Morning). Volicitin and JA both induced increases in β-caryophyllene, (E)-α-bergamotene, and (E)-β-farnesene; however, the kinetics of induced volatile release differed. JA promotes a sustained release of combined sesquiterpenes, with over 20% of the combined sesquiterpenes emitted in the third volatile collection period. In contrast, levels of volicitin-induced sesquiterpenes decline rapidly over time, with less than 8% of the combined sesquiterpenes emitted in the third period.

Leaf damage, produced by mechanical means or insect herbivory, is thought to stimulate a release of free linolenic acid from lipid membranes (Conconi et al. 1996). A portion of the linolenic acid is ultimately converted into cyclopentanone oxylipins, such as 12-oxo-phytodienoic acid and JA, which alter defense-gene expression (Creelman and Mullet 1997). Many elicitors of plant defense responses, are believed to act by influencing JA levels, thus JA is often considered a "master signal" (Gundlach et al. 1992; Muller et al. 1993; Koch et al. 1999). In corn seedlings, mechanical damage alone produces only a minimal increase in volatile sesquiterpenes while the application of beet armyworm (BAW) OS greatly stimulates volatile release (Turlings et al. 1990). Hopke et al. (1994) demonstrated that corn seedlings treated with JA produced both (E)-α-bergamotene and (E)-β-farnesene, which is similar to the response following BAW herbivory (Turlings et al. 1990). Volicitin has been identified as an active elicitor of induced volatiles from BAW OS (Alborn et al. 1997) and, while not yet demonstrated, is thought to also stimulate JA production (Paré and Tumlinson 1999). In this study, JA and volicitin both stimulate sesquiterpene emission; however, compared to JA, volicitin-induced volatile release is more transient. We do not believe that differences in the amounts of volicitin and JA used explain this difference. Our preliminary experiments demonstrated that JA at 5 nmol/plant is not reproducibly active on intact plants (data not shown). Also the single largest volatile release presented in this manuscript (i.e. (E)-β-farnesene) was triggered by volicitin not JA. There are a number of other possible explanations for this apparent discrepancy in volatile-emission kinetics. If volicitin stimulates an endogenous increase in JA, internal and exogenously applied JA pools may be metabolized at substantially different rates. Alternatively, volicitin may preferentially induce 12-oxo-phytodienoic acid, which is known to act on plant responses more rapidly than JA in some systems (Weiler et al. 1993).

Independent of treatment time, volicitin and JA applications on excised leaves released more sesquiterpenes than similarly treated intact plants. This is not

unprecedented, as JA-induced responses have been shown to differ when excised-tissue and intact-plant bioassays are compared. For example, detached barley leaf segments rapidly produce high levels of antimicrobial thionins when treated with JA while intact seedlings exhibit a slower rate of induction (Andresen et al. 1992). Stress following leaf excision may stimulate changes in gene expression. Excision-induced water stress in *Arabidopsis* was shown to significantly increase the expression of over 38% of the 150 mRNA transcripts examined, with significant overlap with wound-induced transcripts. (Reymond et al. 2000). In excised-tissue bioassays, untreated leaves are commonly used as controls to gauge additional treatment effects. Given the observed interaction between excision and elicitation with JA and volicitin, we find that these controls may ignore important physiological changes caused by excision. While our intact-plant bioassays demonstrate that induced plant volatile responses to JA and volicitin are real, they also indicate that excision-induced changes in leaf sensitivity and response can be significant.

A number of possible excision-mediated changes in leaf metabolism could affect volatile release. Leaf detachment may alter either the production of, or sensitivity to, endogenous regulators. In addition to JA, ethylene is known to play a key role in the wound signal transduction leading to proteinase inhibitor (PIN) accumulation in tomato (O'Donnell et al. 1996). Under non-stress conditions, leaf excision has been shown to stimulate increased ethylene production within 24 h (Tsai et al. 1996). In detached plant tissues that are partially water-stressed, ethylene production can be more dramatic (Morgan et al. 1990). Likewise, ethylene perception may change as increased ethylene sensitivity has been proposed to account for jasmonate-induced senescence in excised rice leaves (Tsai et al. 1996). Excision may also act directly on the JA pathway. In *Arabidopsis*, Reymond et al. (2000) demonstrated an increase in both lipoxygenase (LOX2) and allene oxide synthase (AOS) mRNA levels following water stress in detached leaves. Independent of signaling events leading to the induced volatile release, terpenoid metabolism is known to differ in excised and intact tissues. Over a 24-h period, intact and excised peppermint (*Mentha piperita*) plants differentially accumulate ^{14}C-labeled monoterpenes from a pulse of $^{14}CO_2$ (Mihaliak et al. 1991). Excision results in rapid increases and decreases in ^{14}C-labeled monoterpenes while intact plants display a slow and steady increase over time. Mechanistically, this result is not well understood; however, changes in catabolism, conjugation, or volatilization rates are believed to account for these differences. Gershenzon et al. (1993) demonstrated that the metabolic half-life of biosynthesized mono- and sesquiterpenes in excised plants is typically in the order of hours compared to weeks for intact plants. These studies clearly demonstrate that excision greatly changes the metabolic fate of newly synthesized terpenoids. Projections of plant interactions with the biotic environment are commonly made based on experiments with excised tissues (Arimura et al. 2000). We suggest that results from excised-tissue bioassays need to be cautiously interpreted when attempting to extrapolate findings to intact growing plants.

In addition to total quantities, ratios of sesquiterpenes induced by JA and volicitin also differed between excised and intact plants. In JA-treated leaves, excision preferentially increased β-farnesene release at all three treatment times compared to caryophyllene. Trends for (*E*)-α-bergamotene closely match those of (*E*)-β-farnesene, albeit at lower levels. Volicitin-treated plants display a similar trend; however, in the midnight and morning treatments the total ratio of caryophyllene/(*E*)-β-farnesene in both intact plants and excised leaves is lower. The low level of damage-induced sesquiterpenes was essentially unaffected by bioassay design. The altered sesquiterpene ratio suggests that the interaction between elicitation and excision does not act equally on the biosynthesis of individual volatiles. Thus established volatile-release patterns derived solely from excised-tissue studies may not reflect induced-volatile profiles from field-grown plants. This is an important consideration as quantitative, not qualitative, differences in plant volatile profiles are believed to enable parasitoids to discriminate between host and non-host infested plants of the species (De Moraes et al. 1998). It is currently unknown if excision alters induced-sesquiterpene ratios in other species as very few, if any, other studies have directly compared induced volatile release from intact plants and excised tissues.

Many insect-induced volatiles are known to require a latency period between the initial stimulus and the resulting increases in de novo biosynthesis (Loughrin et al. 1994; Röse et al. 1996; Paré and Tumlinson 1997). This delay is likely due to the required activation of metabolic-pathway gene expression (Steele et al. 1998; Frey et al. 2000). *Spodoptera* OS stimulates differential volatile release over time when applied to intact corn seedlings at the beginning of the light cycle (Turlings et al. 1998). While complex, this result is not surprising as both wound- and elicitor-induced defense-gene expression have wide-ranging kinetics (Facchini et al. 1996; Steele et al 1998). What is not well established is how different times of treatment will alter the resulting volatile release in the subsequent photoperiod. In our experiments, volicitin significantly stimulated combined sesquiterpene release above mechanical damage alone when intact plants were treated at Midnight but not Evening and Morning. This result was surprising as intact plants, treated a few hours after the beginning of the light cycle, are routinely used for same-day bioassay purposes (Turlings et al. 1998; and data not shown). This demonstrates that there are different windows of sensitivity in a plant's response to volicitin; however, treatment time does not greatly affect the response to exogenous JA. Volicitin and JA stimulated the greatest release of sesquiterpenes in the Midnight excised leaves; however, the responses of intact plants did not mirror

this trend. While reasons for these differences are unclear, we offer possible explanations for these observed patterns. In undamaged tobacco plants, mRNA transcripts of cysteine proteinases display expression levels that follow a true circadian rhythm (Linthorst et al. 1993). Considering that mRNA levels are highest in the middle of the light cycle and lowest immediately prior to the beginning of the next photoperiod, time of wounding would be expected to influence proteinase accumulation. However, circadian fluctuations in defense- and signal-transduction-related transcript levels in control plants have not been thoroughly investigated. During the Midnight treatment, the corn plants produced guttation droplets, indicative of high positive xylem pressure (Wei et al. 1999). Excision of these leaves would have resulted in a rapid loss in xylem pressure, which may play a role as hydraulic-related signals have been previously implicated in plant defense responses (Malone et al. 1994).

In both intact-plant and excised-leaf bioassays, volicitin and JA stimulate induced sesquiterpene volatile release. Clearly, induced volatile emission in excised tissues is not artifactual; however, the magnitude and ratios of induced volatiles are very different. Due to the pervasiveness of excised-tissue bioassays in the literature, we feel that this is an important consideration. In our experiment the maximum length of time between excision and the beginning of volatile collection was 12 h. Other volatile studies commonly examine leaves 2–7 days after excision (Dicke and Dijkman 1992; Koch et al. 1999). Given this duration for degradative changes to occur, it seems possible that differences in excised leaves and intact plants may be accentuated. Indeed, detached leaves are commonly used as model systems to study leaf senescence (Chang and Kao 1998). The convenience of working with excised leaves ensures their use in the future. However, ease of experimentation has diminished value if similar trends do not exist in both excised tissues and intact plants. Drought-induced ethylene production and the rapid turnover of secondary metabolites are two examples of widely accepted phenomena generated by studies on excised tissues (Apelbaum and Yang 1981; Croteau and Loomis 1972). Currently, both results appear artifactual with regards the physiology of intact growing plants (Morgan et al. 1990; Greshenzon et al. 1993). We have demonstrated volicitin- and JA-induced increases in sesquiterpenes from both intact-plant and excised-leaf bioassays. However, given the interaction between excision and elicitation there needs to be cautious interpretation when projecting the results for excised tissues on to intact-plant models.

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